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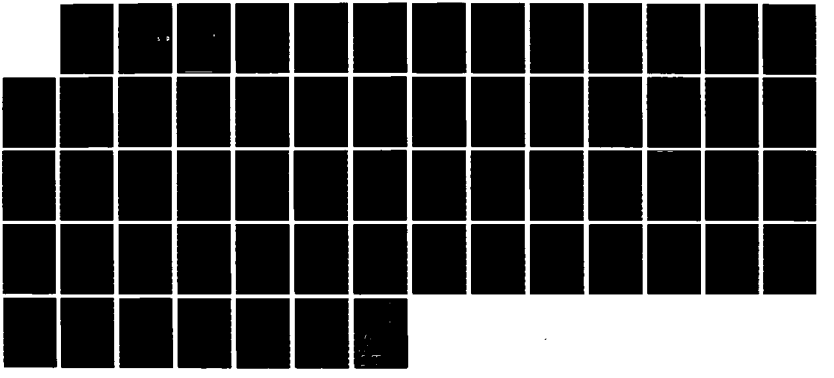

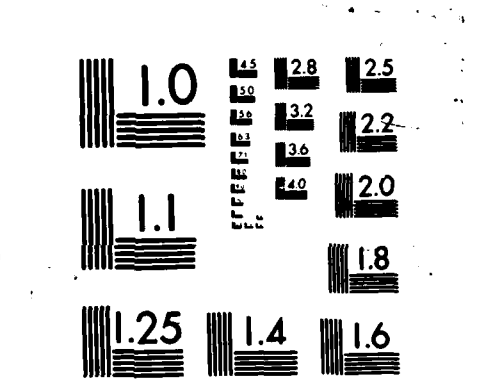
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SULFUR TRANSFERASES AND CYANIDE PRETREATMENT

Annual Report

James L. Way, Ph.D.

September 15, 1985

Support by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This research is concerned with investigating the biochemical and pharmacological properties of the sulfur transferase enzymes in cyanide pretreatment. The enzyme is being encapsulated in erythrocytes with sodium thiosulfate and the toxicologic properties of these procedures are being investigated

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FIGURE 2

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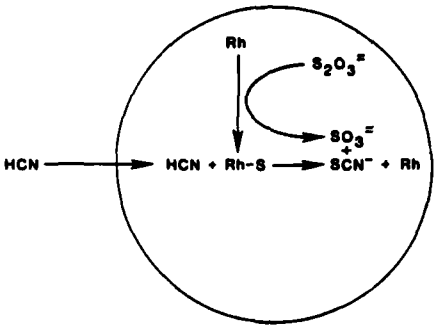

SUMMARY

The predominant mechanism for detoxifying cyanide is to interact cyanide with a sulfur donor, sodium thiosulfate, in the presence of sulfur transferase called rhodanese (Rh). This enzyme is present in the body in large concentrations, has a high turnover number of 20,000, (one molecule of enzyme can metabolize 20,000 molecules of cyanide per minute), and this reaction is irreversible. Therefore, under ideal conditions, a soldier has sufficient enzyme to metabolize over one pound (0.5 kg) of sodium cyanide in five minutes; yet, sodium cyanide is a very toxic chemical and is lethal at one milligram/kg. Therefore, over a finite time period the soldier should be able to metabolize cyanide at a rate of one thousand times a lethal dose per five minutes. Yet, *in reality sodium thiosulfate can protect only two to four lethal doses of* sodium cyanide. The great limiting factor is attributed to the inability of sodium thiosulfate to penetrate cell membrane to sites of rhodanese localization. However, this can be accomplished by encapsulating the rhodanese and sodium thiosulfate together in a red blood cell by hypotonic dialysis (figure 1). Two recent developments permit the application of this approach to development of this new conceptual approach to new antidotes. Firstly, the bovine rhodanese enzyme has a background of information on the three dimensional crystal structure, peptide sequence, amino acid composition and formal enzyme mechanism. Secondly, the technology for encapsulation of materials in red blood cells has vastly improved in the last few years.

Most of the initial first year was spent on attempting to isolate and crystalize this enzyme from beef liver in preparative amounts. Although this enzyme has been crystalized earlier, the preparation of large amounts of the

enzyme involves considerable modification of the procedure. The isolation of rhodanese have been greatly modified. It still involves a series of ammonium sulfate fractionation at various pH values and then this enzyme is subjected to affinity columns employing Cibacron Blue Agar Gel and subsequently DEAE Sephadex A50 anion exchange chromatography. These isolation procedures have been successfully modified so that this enzyme can be prepared in gram quantities.

The erythrocyte encapsulation procedures are being initiated and apparently the encapsulation of this enzyme is feasible. Mouse erythrocytes are isolated by centrifuging in a wash with various buffer solution. The wash red blood cells were subjected to hypotonic dialysis. Subsequently rhodanese and sodium thiosulfate were placed in equilibrium with the erythrocyte and the tonicity of the solution was readjusted to isotonicity. Preliminary studies indicate that radioactive sucrose can be employed as a control marker. Measurements of hematocrit, cell volume, hemoglobin concentration, osmolarity, cell recovery, equilibrium, erythrocyte morphology (light and electron microscopy) are being initiated on the cells. Preliminary studies have been initiated on the <u>in vitro</u> studies to obtain efficient red blood cell loading, to protect the stability of the enzyme during loading procedures and to minimize leakage of the enzyme pyramid. We are still assessing the efficacy of the red blood cell loading and the measurement of the rate of entry and exit of various substrates. The important studies to be done yet are to assess the biochemical function of the encapsulated cells for sodium transport, ability to utilize glucose and ability to metabolize cyanide. These and other functional physiological and biochemical studies are still to be conducted.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Part I

PREPARATIVE ISOLATION OF BOVINE LIVER RHODANESE

ABSTRACT:

1. Rhodanese, thiosulfate-sulfur transferase (EC 2.8.1.1.) was isolated from beef liver by a series of ammonium sulfate fractionations, at low and high pH, affinity chromatography employing Cibacron Blue linked agarose columns and anion exchange chromatography with DEAE Sephadex A50. Two forms of fully active rhodanese with molecular mass of approximately 36,000 daltons were isolated.

INTRODUCTION

Rhodanese, a thiosulfate sulfur transferase (EC 2.8.1.1.) is a major cyanide detoxifying enzyme. This enzyme has an almost ubiquitous distribution in animal tissues. The enzymatic activity varies widely from organ to organ and in different animal species (Lang, 1933; Saunders & Himwich, 1950; Sorbo, 1975; Bernard et al., 1947; Himwich & Saunders, 1948)[1,2,3,4,5]. This enzyme was first crystallized in 1953 (Sorbo, 1953)[6] and a molecular weight of 37,100 was reported (Sorbo, 1953)[7]. Various laboratories have confirmed the molecular weight of this enzyme; however, other studies suggested that rhodanese is a dimer with two identical or similar subunits of molecular mass 18,500 daltons (Volini et al., 1967; Horowitz & Westley, 1970; Volini et al., 1978)[8,9,10], and indicated that enzymatically active fragments of rhodanese may exist (Horowitz & Falksen, 1981)[11].

Beef liver rhodanese has been sequenced, crystallized and its structure determined by X-ray diffraction (Ploegman et al., 1978; Ploegman et al., 1978)[13]. However, in the course of sequence studies of beef liver rhodanese, Blumenthal and Heinrikson (1971)[14] found that the crystalline bovine rhodanese obtained by ammonium sulfate fractionation at low and high pH values, yielded two enzymatically active components when analyzed by anion exchange chromatography. Further investigation in both amino acid composition and in sequence studies showed no difference between the two forms. More recently, heterogeneity has been reported in crystalline bovine liver rhodanese by other laboratories (Canella et al., 1981)[15].

EXPERIMENTAL PROCEDURES

Enzyme Isolation: Rhodanese was isolated from bovine liver by combining and modifying previously reported procedures (Blumenthal & Heinrikson, 1971; Horowitz, 1978)[14,16]. Approximately 4 to 5 Kg of fresh beef liver was frozen (-20°C) and thawed two times to enhance the lysis of cells. Ammonium sulfate fractionations at low and high pH and the use of Cibacron Blue agarose (Bio-Rad, Richmond, CA) were conducted as previously described (Horowitz, 1978)[16] with the modifications described below.

The Cibacron Blue agarose gel was equilibrated with 10 mM sodium phosphate buffer (pH 7.2) and eluted with 0.005 M sodium phosphate containing 0.075 M sodium thiosulfate (pH 7.2) (Horowitz, 1978)[16] using a larger 100 x 2.5 cm^2 column with a 600 ml bed volume. The fractions were monitored at 280 nm (LKB 2138 Uvicord S UV monitor) and collected (LKB 2111 MultiRac fraction collector). It was necessary to repeat the affinity chromatographic procedure two times, as rechromatography resulted in considerable purification of the enzyme.

The enzyme then was concentrated in an Amicon Stirred Cell using a PM10 filter (Amicon Corp., Danvers, MA), and dialysed against 0.01 M sodium phosphate buffer (pH 7.2) before being rechromatographed. After the second passage of the enzyme through the agarose immobilized Cibacron Blue column, the enzyme preparation was no longer colored but still contained a considerable amount of contaminant proteins. These latter proteins can be removed by the method of ammonium sulfate fractionation (Canella et al., 1981; Horowitz, 1978)[15,16] or by further chromatographic fractionation using a DEAE Sephadex A50 anion

exchange column (Blumenthal & Heinrikson, 1971)[14]. The chromatographic procedure usually was employed, as it can resolve the two main rhodanese fractions. The DEAE Sephadex A50 was equilibrated in 5 mM Tris-Sulfate buffer (pH 7.5) containing 10 mM $Na_2S_2O_3$ and placed in a chromatographic column (2.6 x 70 cm x 1.3 cm^2 Pharmacia K26/70). The enzyme solution was precipitated in 2.5 M ammonium sulfate and suspended and dialysed in the gel equilibration buffer before loading onto the anion exchange column. A gradient elution of 200 ml of 10 mM $Na_2S_2O_3$ in the mixer flask and 1500 ml of 20 mM $Na_2S_2O_3$ in the reservoir flask in 5 mM Tris-Sulfate pH 7.5 clearly resolved two rhodanese containing fractions. The enzymes contained in each of the two rhodanese fractions were pooled and concentrated separately. Each fraction then was equilibrated in 10 mM sodium phosphate buffer containing 1 mM $Na_2S_2O_3$ at pH 7.2. The specific activities of these peaks, hereafter referred to as rhodanese I and rhodanese II respectively, were determined by the procedure of Wang and Volini (1968)[18] and exceeded 200 M SCN^- per mg protein per min. To facilitate the description of the electrophoretic patterns obtained from limited proteolytic digests where both algebraic and alphabetical notations are used, it was more convenient to refer to these enzyme forms as rhodanese I and rhodanese II rather than A and B as use by Blumenthal and Heinrikson (1971)[14] even though they are probably the same enzymes. The enzyme solutions were stable up to 10 mg/ml in 10 mM sodium phosphate (pH 7.2) buffer containing 1 mM $Na_2S_2O_3$ when stored at -70°C. Rhodanese was not stable when stored without sodium thiosulfate.

Activity of Rhodanese: Rhodanese activity was measured from the rate of formation of thiocyanate (Wang & Volini, 1968)[18]. The concentration of the ferric iron-thiocyanate complex was measured spectrophotometrically at 460nm.

Protein Concentrations: Protein concentrations were determined by the Bio-Rad protein assay method (Bio-Rad Laboratories, Richmond, CA). This method was initially calibrated by the Lowry procedure (Lowry et al., 1951)[19]. The concentration of pure rhodanese agreed with its molecular extinction coefficient: E_{280}=17.5 (Sorbo, 1953)[7].

Sedimentation Coefficients and Molecular Weights: The sedimentation coefficients were determined on a Model E analytical ultracentrifuge at the Bioanalytical Laboratories of Washington State University. The values for rhodanese I and rhodanese II proteins in 0.01 M sodium phosphate were 2.81 and 2.84 respectively. Since these values were obtained at the relatively low enzyme concentration of 2.6 and 4.6 mg/ml, the S values are slightly lower than those of S = 3.0 and 2.9 reported by Sorbo (1953)[7] and S = 3.2 reported by Green and Westley (1961)[20]. The values are not corrected for protein concentration or viscosity.

The molecular mass was determined by the Meniscus Depletion Sedimentation Equilibrium Method (Chervenka, C.H., A Manual of Methods for the Analytical Ultracentrifuge, Spinco Division of Beckman Instruments, Inc., Palo Alto, CA)[21]. The molecular mass was determined at about 1mg/ml in 10 mM sodium phosphate pH 7.2 buffer. The values obtained for rhodanese I and II are 36,600 and 36,300 daltons. This is in close agreement with molecular weights of 35,000

to 37,000 determined by other workers (Sorbo, 1953; Horowitz & Westley, 1970)[7,9].

RESULTS

Rhodanese has been purified from bovine liver by combining procedures developed by previous workers (Blumenthal & Heinrikson, 1971; Horowitz, 1978)[14,16] with modifications. In preparative isolation of rhodanese, affinity chromatography with Cibacron Blue linked agarose (Horowitz, 1978)[16] yielded a discolored product, therefore, it was necessary to rechromatograph this fraction again. This procedure yielded a product which contained about 50% non-rhodanese proteins. It should be pointed out that our modifications deleted various steps from an earlier procedure (Horowitz, 1978)[16] and added an anion exchange chromatographic step (Blumenthal & Heinrikson, 1971)[14].

The final purification of rhodanese by other laboratories usually was conducted either by a series of ammonium sulfate fractionations with pH manipulations (Horowitz, 1978)[16] or by the passage of the enzyme preparation through a DEAE Sephadex A50 column (Blumenthal & Heinrikson, 1971)[14]. The former procedure yielded a crystalline product which, in our hands employing SDS electrophoresis, usually exhibited multiple protein bands. The gradient elution of rhodanese from the DEAE Sephadex A50 column in our procedure usually yields two major fractions of rhodanese, rhodanese I and rhodanese II. The rhodanese from each peak was found to be electrophoretically homogenous. This final highly purified fraction has been crystallized by us, but the enzyme fractions usually were concentrated and stored in solution at 1-10 mg/ml in 10 mM sodium phosphate buffer (pH 7.2) containing 1mM sodium thiosulfate. This material was stable when

stored at -70°C over many months. A typical isolation of bovine liver rhodanese is shown in Table 1.

The hydrodynamic properties of the two forms of rhodanese were similar with S values of 2.81 and 2.84 and molecular weights of 36,600 and 36,300 for rhodanese I and II respectively. These are in reasonable agreement with the values obtained by other workers (Sorbo, 1953; Volini et al., 1967)[7,8]. In rhodanese prepared by the above procedure, a low molecular weight rhodanese fraction was not observed. (Volini et al., 1967; Volini et al., 1978)[8,10].

The SDS polyacrylamide gel electrophoresis of the two forms of rhodanese showed a slight but reproducible difference in their electrophoretic mobility (see fig. 1). Rhodanese I consistently moved slower than rhodanese II.

DISCUSSION

The purification of liver rhodanese on a preparative scale dictated modification of presently available methods. The substitution of some of the ammonium sulfate fractionation steps with Cibacron Blue linked agarose provided a rapid convenient method for the resolution of bovine beef liver rhodanese (Horowitz & Falksen, 1981)[11]. Further improvement in reliability and reproducibility was obtained by recycling the enzyme through the Cibacron Blue linked agarose gel again plus the addition of Sephadex A50 anion exchange chromatography. The binding of rhodanese to immobilized Cibacron Blue F3GA may not be attributed to a nucleotide site, as the binding of many proteins to Cibacron Blue linked agarose is mediated by less specific hydrophobic and electrostatic protein-dye interaction (Reisler & Liu, 1981)[23] and not necessarily through a dinucleotide-fold specific site as proposed earlier (Thompson et al., 1975)[24].

This study confirms the earlier studies (Blumenthal & Heinrikson, 1971)[14], that two forms of rhodanese were present. Their sequence and amino acid analysis showed no difference in the two forms. Our amino acid analysis is consistent with peak I having less negative charge as it contained more ammonia in peak I rhodanese than that from peak II. In addition, rhodanese obtained by a second procedure, extensive dialysis followed by passage through Bio-Gel P-2 polyacrylamide also showed rhodanese I to have more ammonia than rhodanese II.

Earlier, SDS polyacrylamide gel electrophoresis on rhodanese (Canella et al., 1981)[15] showed only one molecular form of rhodanese. However, these electrophoretic studies were performed in tubes where

small molecular weight differences would not be apparent. However, differently charged species of rhodanese were also reported from non-detergent polyacrylamide gel electrophoresis (Canella et al., 1981)[15]. These were attributed to differences in cyanolysible sulfur.

In these studies, the molecular weights of rhodanese I and II were consistently different with rhodanese I migrating somewhat slower than rhodanese II in SDS polyacrylamide gel.

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Table I Purification of Bovine Liver Rhodanese

Fraction	Rhodanese Units	Protein mg	Specific Activity
Extract	671,600	1,196,000	0.56
0.4 M $(NH_4)_2SO_4$, pH 3.8	782,000	333,500	2.3
1.4 M $(NH_4)_2SO_4$	599,000	188,300	3.2
2.0 M $(NH_4)_2SO_4$ Supernatant	437,000	42,400	10.3
Cibacron Blue (1)	210,000	3,800	55.3
Cibacron Blue (2)	185,000	2,200	84.1
2.5 M $(NH_4)_2SO_4$ Precipitate	158,000	1,700	92.9
DEAE Sephadex A-50			
Rhodanese I	54,400	264	205
Rhodanese II	86,800	399	217

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Fig. 1. SDS polyacrylamide slab gel electrophoresis of the two forms of beef liver rhodanese resolved by DEAE sephadex A50 anion exchange chromatography.

Channel 1, rhodanese I; channel 2, rhodanese II; channel 3, standard ribosomal protein mix. See text for experimental details.

Part II

RED BLOOD CELL CARRIER MECHANISM FOR RHODANESE

ERYTHROCYTE ENCAPSULATED THIOSULFATE SULFURTRANSFERASE

The mechanism of cyanide intoxication is attributed to be the inhibition of the terminal oxidase on the mitochondrial respiratory chain, cytochrome oxidase (1, 2). Although other enzymes probably are involved in massive poisoning, this represents the most sensitive enzyme and is believed to contribute predominantly to its toxicity. One of the antidotes for cyanide poisoning is sodium thiosulfate (3). This substrate acts as a sulfur donor in the presence of the sulfurtransferase, rhodanese, to rapidly metabolize cyanide to the less toxic thiocyanate (4).

Although sodium thiosulfate is a very effective cyanide antidote, from a biochemical viewpoint it should be far more effective, as rhodanese is present in very high concentrations in various organs throughout the body and it has a high turnover number (5, 6). It is estimated that,under ideal conditions, one to two moles of sodium cyanide can be metabolized per minute. Since a lethal dose of cyanide in man has been estimated to be 1 to 2 mg/kg, it is apparent that if the sulfur donor and the enzyme can be operating under more optimal conditions, a formidable antidote to cyanide can be developed. This is not observed partly because of the physiological disposition of these reactants. Sodium thiosulfate is a highly ionized inorganic molecule which penetrates cell membranes very slowly; therefore, it would have a limited intracellular distribution. Since the enzyme for detoxifying cyanide, rhodanese, is present predominantly intracellularly in the mitochondria, the sodium thiosulfate would not distribute to sites of toxicant (HCN) nor to sites of localization of the enzyme which is involved in cyanide detoxification. Therefore, these preliminary studies represent the initial attempt to facilitate the tissue

localization of a sulfur donor and sulfurtransferase to the same tissue localization in order to develop a more effective cyanide detoxication mechanism. Either thiosulfate must be facilitated to sites of enzyme distribution or vice versa. Although the administration of free rhodanese can enhance the antidotal potency against cyanide, this application is of limited value because of unfavorable biological disposition and immunological factors (7).

The approach presented herein is to encapsulate highly purified bovine rhodanese and sodium thiosulfate into erythrocytes to provide a means to rapidly detoxify cyanide. Conceptually, this would provide a different approach to drug antagonism. The sulfur donor (antidote) can be encapsulated with the highly purified detoxifying enzyme in a protective microenvironment which is highly permeable to the toxicant, hydrogen cyanide. This should provide the basis for a highly effective detoxification of cyanide with minimal toxicity from the antidotes.

METHODS OF PROCEDURE

Enzyme Purification

The details on the purification of rhodanese is described elsewhere (5, 8, 9). The purification and crystallization of thiosulfate sulfurtransferase is derived from bovine tissue. A series of extractions, ammonium sulfate precipitation, affinity columns employing Cibacron blue agarose gels and anion exchange chromatography are employed to purify these enzymes. These procedures are initiated by employing 30 pounds of beef liver which are frozen and thawed several times to liberate the enzyme from the mitochondria. The liver is then homogenized with sodium thiosulfate and the homogenate is centrifuged to remove cellular debris and then subjected to a series of ammonium sulfate fractionations at different pH values. Ultimately, the solids are removed and additional ammonium sulfate is added to attain a concentration of 1.4 molar ammonium sulfate and these solids are again removed by centrifugation. A subsequent addition of more ammonium sulfate to 2.0 molar ammonium sulfate will salt out the enzyme. The ammonium sulfate is then removed from the enzyme by dialysis and then further purified by affinity column chromatography employing Cibacron blue agarose gels. The fractions containing the enzymes are collected and further purified by additional affinity column chromatographic procedures. Ultimately, the enzyme is placed on a DEAE sephadex anion exchange column and this resolves the enzyme into two isozymes with the ultimate purification of approximately 600 fold. It is this highly purified enzyme which is employed in our red blood cell encapsulation studies, as a high specific activity is necessary to initiate these studies.

ENCAPSULATION PROCEDURE

Whole blood was obtained from adult male Balb/C mice (20-30 gm) by cardiac puncture. After collection, plasma and white cells were removed by centrifugation and the red cells were resuspended and washed three times with an isotonic phosphate-buffered solution. The ratio between buffer and red blood cells was maintained at 10:1 (v/v). Hypotonic dialysis of the prewashed erythrocytes were then carried out with minor modifications (10, 11, 12, 13). Dialysis of red blood cells was conducted for 30 minutes at 2°C in a hypotonic buffer. The predialyzed erythrocytes were then mixed for 30 minutes at room temperature (25°C) with either ^{14}C-sucrose or rhodanese, ATP and $Na_2S_2O_3$. Subsequently, the erythrocytes were resealed by incubating for 30 minutes at 37°C in a hypertonic phosphate buffer. The cells were then washed 3 times with an isotonic phosphate-buffered solution. Throughout the encapsulation process, red blood cell counts and hematocrit determinations were performed as indicated in the schematic. Carbon 14 - sucrose was purchased from New England Nuclear Corporation, (Boston, MA) and all chemicals employed met ACS specifications.

Thiocyanate formation in loaded red blood cells was performed in the following manner: an aliquot (10 ml) of loaded red blood cells was suspended in an isotonic buffered-solution containing KCN and $Na_2S_2O_3$ 5 H_2O and the formation of thiocyanate was determined at 37°C. In comparative studies conducted without the red blood cell the content of the incubation mixture was identical other than the fact that the red blood cell was not included. The amount of rhodanese, cyanide and thiocyanate were determined as previously described. In the control studies with radioactive sucrose, the LS 7500 liquid-scintillation counter was employed. Aliquots of loaded red cells were

applied to filtered disks to absorb the hemoglobin in order to minimize quenching and allowed to dry overnight. Water (0.5 ml) was added to each dried disc and counted with Bray's scintillation fluid.

RESULTS AND DISCUSSION

Mouse erythrocytes were dialyzed in a hypotonic media to increase the percentage of the entrapped enzyme, as this sulfur transferase requires a rather long, tedious procedure to be obtained in the highly purified crystalline state. The erythrocytes were predialyzed in a hypotonic media and subsequently the enzymes were placed in the dialysis bag with the red blood cells so that the degradation of the enzyme will be minimized. The initial hematocrit of this preparation is over 60%. This is assuming that the enzyme will be fully equilibrated between the intracellular and extracellular spaces and irreversible lysis of the cells is minimal.

The initial studies were conducted with carbon-14 labeled sucrose. The control employed in these studies used isotonic rather than hypotonic saline. These encapsulation procedures resulted in a 30% encapsulation of the added radioactive glucose with a 72 - 95% recovery of the erythrocytes. The hematocrit after encapsulation was decreased and this would be consistent with the studies by other laboratories (7) that the animal erythrocytes loaded by dialysis were smaller in size than the untreated red blood cells (Table III). Of considerable importance is the effect of sodium thiosulfate on the encapsulation procedure, as this serves as the sulfur donor for the detoxification of cyanide. Sodium thiosulfate, in the concentration employed in these studies, had no effect on the encapsulation of carbon-14 labeled sucrose, as there was no significance difference between encapsulation studies conducted with and without sodium thiosulfate (Table I). In the in vivo survival of the free and erythrocyte encapsulated sucrose (Table II) the half life of the free sucrose was approximately 1/2 hrs. During the first 60 min

there was a 48% loss of sucrose-loaded erythrocyte. In the next 48 hrs the sucrose-loaded erythrocytes decreased by approximately 15%. This is consistent with other studies where the sucrose-loaded erythrocytes showed an initial rapid destruction, and this is subsequently followed by a second decay curve where the disappearance of the sucrose-loaded erythrocytes from the circulation was much slower. The half life of the second phase was approximately 10 days. These biphasic disposition curves for the survival of sucrose-loaded erythrocytes are consistent with other studies, as it has been reported that 25 - 40% of the sucrose-loaded erythrocytes can be removed from the circulation during the initial 12 - 24 hrs (7). These in vivo survival studies were critical in the long term objective, as the ultimate aim was not only to obtain maximal loading of the enzyme and substrate, but also to obtain a high percentage of survival of the red blood cells. Because of these aims, the procedures were modified to cause minimal damage to the red blood cells in order to minimize the removal of these damaged cells by the reticular endothelial system. These data seem to suggest that in the resealing procedure of the red blood cells the damage was minimal with regard to making the red blood cells more susceptible to rapid removal from the circulation.

Since in vivo survivability of carbon-14 sucrose encapsulated in the red blood cells was satisfactory, studies then were instituted comparing the erythrocyte entrapment with other compounds (Table III). With an increase in molecular weight, the erythrocyte encapsulation of these same compounds appears to be enhanced. Sucrose was entrapped approximately 30%, inulin 41% and rhodanese 69%. The variations in the percent encapsulation of rhodanese varied between 30 to 69%. In these experiments (Table IV) approximately 69% of the added enzyme was recovered in the mouse erythrocyte. The enzyme appears to be

fully equilibrated (98%) as a concentration of 16.4 rhodanese units/ml of rhodanese was present in the extracellular space and in the erythrocytes. Over 95% of the cells were recovered by these procedures and the hematocrit found to decrease from 60 to 45% in the hypotonic dialysate, whereas in the control studies there was no change in the hematocrit. This would be consistent with other studies showing that hypotonic dialysis can result in a decrease in size of the erythrocytes (7). In in vitro studies these rhodanese-thiosulfate-encapsulated red blood cells were found to rapidly convert cyanide to thiocyanate, and the turnover number of the encapsulated enzyme substrate incubation mixture was approximately 500. These in vitro studies reflect the potential of utilizing these encapsulated enzyme-substrate preparations in drug antagonism studies.

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TABLE I

RBC ENCAPSULATION

	PERCENT ENCAPSULATION	
		$Na_2S_2O_3$
^{14}C-SUCROSE	28.5 ± 4.1	30.1 ± 1.8

TABLE II

INVIVO SURVIVAL OF SUCROSE-LOADED MURINE ERYTHROCYTES

TIME (hours)	SURVIVAL (%)
0	(100)
1/2	57.50 ± 0.00
1	51.25 ± 0.45
2	48.55 ± 2.15
4	47.50 ± 0.60
8	43.75 ± 0.75
17	42.95 ± 0.85
24	39.35 ± 0.85
48	36.55 ± 1.05

TABLE III

RBC ENCAPSULATION

	Per Cent Encapsulation
^{3}H Inulin	41.2
^{14}C Sucrose	30.1 ± 1.8
Rhodanese	68.9 ± 2.1

TABLE IV

ENCAPSULATION OF RHODANESE IN MURINE ERYTHROCYTES

	Murine erythrocytes	
	Dialyzed	Control
Encapsulation (%)	69.0	4.8
Equilibrium (%)	100.0	28.8
Recovery of cells (%)	95.2	99.1
Initial hematocrit (%)	60.0	64.0
Final hematocrit (%)	44.7	62.6

Part III

ENCAPSULATION OF THIOSULFATE: CYANIDE SULFURTRANSFERASE BY MOUSE ERYTHROCYTES

ABSTRACT

Encapsulation of Thiosulfate:Cyanide Sulfurtransferase by Mouse Erythrocytes.

Murine carrier erythrocytes, prepared by hypotonic dialysis, were employed in the encapsulation of several compounds including ^{14}C-sucrose, ^{3}H-inulin and bovine thiosulfate:cyanide sulfurtransferase (rhodanese), a mitochondrial enzyme which converts cyanide to thiocyanate. Approximately thirty per cent of the added ^{14}C-sucrose, ^{3}H - inulin, and rhodanese were encapsulated by predialyzed erythrocytes, and a decrease in the mean corpuscular volume and mean corpuscular hemoglobin were observed. In the encapsulation of rhodanese a recovery of 89% of the erythrocytes was achieved and an 87% equilibrium was established. The addition of potassium cyanide (50 mM) to intact, rhodanese-loaded erythrocytes containing sodium thiosulfate resulted in its metabolism to thiocyanate. These results establish the potential use of erythrocytes as biodegradable drug carrier in drug antagonism.

INTRODUCTION

The toxic effects of cyanide have been attributed to the inhibition of cytochrome oxidase, the terminal oxidase of the mitochondria respiratory pathway (Keilin, 1929; Warburg, 1931), resulting in the production of a histotoxic anoxia. Sodium thiosulfate, one of the antidotes employed in treating cyanide toxicity, is a sulfur donor for thiosulfate:cyanide sulfurtransferase (EC2.8.1.1), which is also known by its trivial name of rhodanese, a mitochondrial enzyme responsible for biotransforming cyanide to thiocyanate (Lang, 1933; Himwich and Saunders, 1948). This enzyme has a turnover number of 20,000 min^{-1} (Sorbo, 1953; Westley, 1981); therefore, under ideal <u>in vitro</u> conditions, one mole of rhodanese can detoxify 20,000 moles of cyanide per minute. Since the intracellular distribution of rhodanese is mitochondrial, the efficacy of endogenous rhodanese to detoxify cyanide is limited even though it is present in large amounts, as sodium thiosulfate does not readily penetrate cell membranes. Therefore, sodium thiosulfate would neither distribute to the sites of cyanide localization nor to sites of rhodanese distribution. This would partially explain why sodium thiosulfate does not attain its theoretical potential against the lethal effects of cyanide.

These studies were conducted in an attempt to circumvent some of these adverse disposition factors by placing the enzyme and sulfur donor in close proximity to each other in a protective environment which is still readily accessible by the toxicant, hydrogen cyanide, for detoxification. The approach, as depicted in figure 1, is to encapsulate rhodanese with sodium

thiosulfate in an erythrocyte, which would function as a biodegradable drug carrier. The carrier erythrocyte, is highly permeable to cyanide under physiological conditions, as the pKa of cyanide is 9.2, and also it would provide a protective environment for the detoxifying enzyme and its substrate. Although encapsulation of exogenous enzymes or drugs in intact erythrocytes have been reported to serve as a potential vehicle for the delivery of therapeutic agents (Ihler et al., 1973; Deloach et al., 1980a; Fiddler et al., 1980; Pitt et al., 1983), these studies represent the first attempt to use carrier erythrocytes encapsulated with enzymes and substrates in an attempt to antagonize an exogenous chemical toxicant.

Materials and Methods

Animals. Balb/c male mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing between 18-30 g, were housed in temperature (22-24°C) and light-controlled rooms, were maintained on Rat Chow 5012 (Ralston Purina Co., St. Louis, MO) and water, ad libitum.

Chemicals and isotopes. Carbon 14 labeled sucrose (673mCi/mmole) was purchased from New England Nuclear Corporation (Boston, MA). Tritiated inulin (1.84Ci/mmole) was obtained from Amersham Corporation (Arlington Heights, IL). Cibacron blue agarose gel is a product of BioRad (Richmond, CA.) All other chemicals employed were of the highest purity commercially available.

Isolation and Purification of Rhodanese. All isolation and purification procedures were conducted at 2-5°C as described by Sorbo (1953) and Westley (1981) with modifications for preparative procedures. Fresh bovine liver (approximately 14 kg.) were sliced one inch thick, frozen and thawed several times. After refreezing, the liver was divided into three aliquots, homogenized with 1.5 volume of cold 10 mM sodium thiosulfate, and centrifuged (27,578g x 60mins) to remove cellular debris. Supernatant fluids were combined and subjected to successive ammonium sulfate (0.4M), pH(3.8), and ammonium sulfate (1.4M) fractionation procedures and the precipitate was removed by centrifugation (27,578g x 60mins). Subsequently, ammonium sulfate was added to the supernatant fraction to bring the final concentration to 2.0M and the preparation was allowed to stand overnight. The pelleted enzyme was collected by centrifugation (25,578g x 30min), dialyzed, and subjected to column chromatography with Cibacron blue agarose gel (10 cm x 5 cm^2). The sample is

applied and washed with 10mM sodium phosphate buffer (pH=7.2), eluted with 10mM sodium phosphate buffer containing 75mM sodium thiosulfate (pH=7.2), and concentrated in an Amicon stirred cell using a Diaflo PM10 filter. The concentrated sample is then subjected to a series of pH-ammonium sulfate fractionation and precipitated overnight. White crystalline enzyme was obtained by recrystallization in 1.8 M ammonium sulfate. The purification steps resulted in an approximate 600 fold increase in purity with about 9-10% recovery. It is this highly purified enzyme which is employed in our erythrocyte encapsulation studies. One unit (U) of rhodanese is defined as that amount of enzyme which catalyzed the production of one micromole of thiocyanate per minute (Westley, 1981).

Erythrocyte Encapsulation. Whole blood was obtained from adult male Balb/c mice under anesthesia (diethyl ether) by cardiac puncture with heparinized syringes. After collection, plasma and white cells were removed by centrifugation and the pelleted red blood cells were resuspended and washed three times with an isotonic phosphate-buffered solution(144mM NaCl, 2mM $MgCl_2.6H_2O$, 5mM dextrose, pH=7.4, 304mmol/kg). The ratio between buffer and red blood cells was maintained 10:1 (v/v). Hypotonic dialysis of the prewashed erythrocytes then was conducted by method of Deloach et al. (1980b), with minor modifications. Dialysis of red blood cells was conducted for 30 minutes at 2°C in a hypotonic phosphate-buffered saline (4mM $MgCl_2.6H_2O$,5mM dextrose, pH=7.3, 45mmol/kg). The predialyzed erythrocytes were then mixed for 30 minutes at room temperature (25°C) with either ^{14}C-sucrose, ^{3}H-inulin or rhodanese (30-40 units, 65 U/mg). Subsequently, the erythrocytes were resealed and annealed by incubating for 30 minutes at 37°C in a hypertonic phosphate buffered saline (450mM NaCl,10mM Na_2HPO_4/NaH_2PO_4,pH=7.3, 852 mmol/kg). The

cells were then washed three times with an isotonic phosphate-buffered solution. Hematologic determinations including red blood cell counts, mean corpuscular volume (mcv) in femtoliters ($fl=1x10^{-12}ml$) and hematocrit (Hct) were performed with a Coulter Counter in conjunction with a MCV/HCT/RBC computer(Curtis Matheson Scientific, Inc., Houston, TX). Osmolarity (mmol/kg) was ascertained with a vapor pressure osmometer (Wescor, Inc., Logan, UT). The mean corpuscular hemoglobin content (mchb) in picograms(pg) was calculated by dividing the weight of hemoglobin by the number of red blood cells in a unit volume of blood. Radioactivity in blood samples was measured by counting aliquots in Bray's scintillation fluid in a LS 7500 liquid-scintillation counter (Beckman Instruments, Irvine, CA). The percentage of rhodanese or ^{14}C-sucrose encapsulated was calculated from the amount encapsulated divided by the amount added times 100. Percentage of equilibrium was obtained from the concentration of ^{14}C-sucrose or rhodanese inside the erythrocytes divided by the concentration of ^{14}C-sucrose or rhodanese outside the erythrocytes during the mixing phase. The percentage of cells recovered was calculated from actual cell counts performed with a Coulter Counter.

Thiocyanate in loaded red blood cells was determined by suspending an aliquot (10 ul) of loaded red blood cells in an isotonic-buffered solution containing 50 mM KCN and 50 mM $Na_2S_2O_3$ 5 H_2O and the formation of thiocyanate at 37^oC was measured spectrophotometrically (Westley, 1981) at periodic time intervals. All experiments were performed at least two to three independent times and the results were expressed as mean $\pm$ 1 SEM.

RESULTS

To ascertain that the encapsulation technique is consistent with other laboratories, ^{14}C-sucrose was employed as a controlled marker in the balb/c mouse erythrocytes. In an attempt to establish optimal conditions in preparing murine carrier erythrocytes, washed erythrocytes were dialyzed against a hypotonic saline solution and its osmolality (Figure 2a) and percent encapsulation of ^{14}C-sucrose (Figure 2b) were determined. A reduction in the osmolality of the erythrocytes occurs quite rapidly (Figure 2a) and thirty minutes of dialysis is sufficient to attain maximum encapsulation of ^{14}C-sucrose (Figure 2b). Since dialysis in excess of thirty minutes resulted in a decrease in the percentage of encapsulation, a 30 minute dialysis time interval was employed for all subsequent experiments. Approximately 30% of the ^{14}C-sucrose and ^{3}H-inulin can be encapsulated (Table 1). Radioactive ^{3}H-inulin was employed as a larger molecular weight marker, since it should behave more similar to that of rhodanese.

During the preparation of carrier erythrocytes, several hematological indices were monitored to compared sucrose-loaded erythrocytes with control-treated erythrocytes (Table 2). After encapsulation, the mean corpuscular volume (mcv) of the sucrose-loaded erythrocytes was reduced by 14% (Table 2) when compared to control-treated erythrocytes(40fl). Similarly, the rhodanese-loaded erythrocytes had a smaller mcv (Table 3) when compared to control-treated erythrocytes. With a reduction in the mcv, there was an associated decrease in hemoglobin. Approximately 55% of the hemoglobin was retained after encapsulation (Table 2). The cell recovery and equilibrium achieved in the encapsulation process were adequate. In the controlled studies where the

erythrocytes were dialyzed against isotonic saline solution, less than 1% of the ^{14}C-sucrose was encapsulated but the cell recovery is almost complete (Table 2). The low percentage of equilibrium suggests that most of the ^{14}C-sucrose is still extracellular.

Other studies employing rhodanese demonstrate that 30% of the rhodanese (Table 1) was encapsulated and changes in the mcv, mchb, and cell recovery after encapsulation were similar to those obtained with ^{14}C-sucrose. It should be noted that in control studies, 4.6% of the rhodanese was "encapsulated" by erythrocytes which were predialyzed against isotonic saline. This amount is slightly higher than expected. Again, there is no difference in the percentage of cells recovered in either the dialyzed or control cells.

To evaluate the potential ability of the encapsulated enzyme to metabolize cyanide, in vitro studies employing erythrocytes loaded with rhodanese and thiosulfate were conducted. Subsequent to the addition of potassium cyanide to the above loaded erythrocytes, the metabolism of cyanide is quite evident as demonstrated by the formation of thiocyanate (Figure 3).

DISCUSSION

These studies have conceptual significance to toxicology, as it probably represents the first attempt to employ erythrocyte as a drug carrier mechanism in the antagonism of exogenous chemical toxicants. The application of erythrocytes as a carrier is relatively recent, as it was only reported in 1979 (Ihler) that with hypotonic dialysis, the erythrocytes become swollen with the development of numerous transient openings (Ihler, 1979; Seeman, 1967; Baker, 1967) of sufficient size to permit macromolecules such as hemoglobin to escape (Baker, 1967). This then provides the basis to incorporate various substances into the red blood cells, as equilibrium can be readily established, resulting in the diffusion of intracellular contents out of the cell and large extracellular molecules into the cell.

Thiosulfate, as a sulfur donor, should be a more effective antagonist against the lethal effect of cyanide because of the high content and turnover number of rhodanese. The inability of sodium thiosulfate to achieve its maximal therapeutic potential is attributed to its limited penetration of cell membranes; therefore, it does not distribute to sites of rhodanese localization. In contrast, cyanide exists predominately in the diffusible, unionized form of hydrogen cyanide, as the pka is 9.2. Therefore it can readily penetrate cell membranes and freely distribute throughout the various biological compartments. By the use of erythrocytes as a drug carrier, it is possible to incorporate rhodanese and sodium thiosulfate into a single compartment where cyanide could readily penetrate the cell membrane in the form of hydrogen cyanide. Moreover, consideration of the vascular compartment as

the site of cyanide detoxication would be consistent with the pharmacokinetic studies previously described where detoxification occurs predominately in the central compartment, which has a volume of distribution equal to that of blood (Sylvester et al., 1984). The potential use of this drug carrier mechanism to detoxify cyanide has important implications, as there is a high content of bovine rhodanese, its turnover number is 20,000 and this reaction is essentially irreversible. Therefore, the encapsulation of the cyanide antagonist provides a unique opportunity to investigate drug antagonism under optimal disposition conditions. If one is to extrapolate the potential of these studies to primates, it would provide a mechanism for a cell to circulate for a few months and its activity to detoxify cyanide, in this case, is latent unless cyanide is present. Under the presence of cyanide, it now becomes a very potent mechanism to rapidly convert cyanide to the much less toxic metabolite, thiocyanate.

The initial studies are being conducted in mice, since the protective effect against the lethality of cyanide is being assessed. From a general concensus, ^{14}C-sucrose has been employed as a standard marker for comparing preparations of carrier erythrocytes from different laboratories. In this regard, our encapsulation techniques apparently have been established, as the various encapsulations and hematological parameters are consistent with reports by other laboratories. Also, tritiated inulin, a larger molecular weight standard, also was employed in our studies, since rhodanese and a low molecular substrate, sodium thiosulfate, are encapsulated. The only discrepancy between the carbon-14 sucrose and the rhodanese incorporation lies in the control studies conducted with isotonic saline. Under these circumstances, the rhodanese is membrane bound in higher amounts to the cell than sucrose, as is

evident in controlled isotonic predialyzed studies. Rhodanese was bound about seven times higher to red blood cell membranes than sucrose. This binding of rhodanese to red blood cells probably does not represent encapsulation, but non-specific binding, as occurs with many proteins, to the red blood cell membrane. With regard to duration of dialysis, control studies indicate that dialysis time should not exceed 30 minutes in the mouse, as the amount of encapsulation rapidly decreases thereafter. These results are consistent with those reported by DeLoach and co-workers (1980b), and this has been attributed to irreversible lyses or rupturing of the erythrocytes after prolonged dialysis. The hematological studies indicate that the mcv and mchb decreases after red blood cell encapsulation. The mechanism underlying this decrease in mcv is not clear. However, it would seem reasonable that the loss of cellular content during hypotonic dialysis may account for the reduction of the mean corpuscular volume, as well as hemoglobin concentration.

Once the enzymes and substrates have been encapsulated into red blood cells, it is important to assess the functional capacity of these encapsulated erythrocytes in performing normal metabolic function as well as specialized toxicologic function. These encapsulated cells appear to retain the anticipated capability of catalyzing the biotransformation of cyanide. This then represents a potential vehicle for the delivery of therapeutic agents and would protect them from premature degradation, inactivation, or excretion with subsequent loss of pharmacologic activity. A sustained level of the detoxifying enzyme and substrate is of considerable potential as, on a theoretical basis, this unique drug carrier erythrocytic mechanism provides the

basis to antagonize the lethal effects of cyanide in a magnitude that far exceeds any methods presently known.

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FIGURE LEGEND

Figure 1. Erythrocyte encapsulation of rhodanese (Rh) and sodium thiosulfate.

Figure 2a. Osmolality of the erythrocytes during hypotonic dialysis. Erythrocytes were dialyzed against a hypotonic saline solution.

Figure 2b. Relationship of duration of dialysis and percentage of encapsulation. Aliquots of erythrocytes were removed during hypotonic dialysis and equilibrated with ^{14}C-sucrose.

Figure 3. Thiocyanate formation in erythrocytes containing rhodanese (8.25$\pm$ 0.23 units/ml erythrocyte) and sodium thiosulfate following the addition of potassium cyanide.

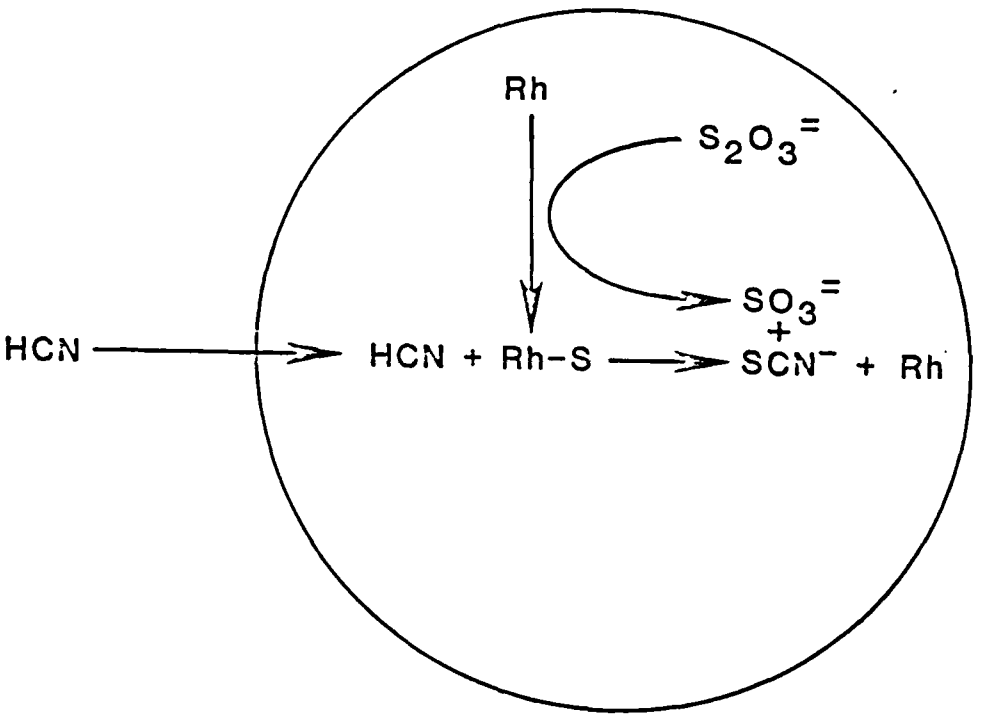

FIGURE 1

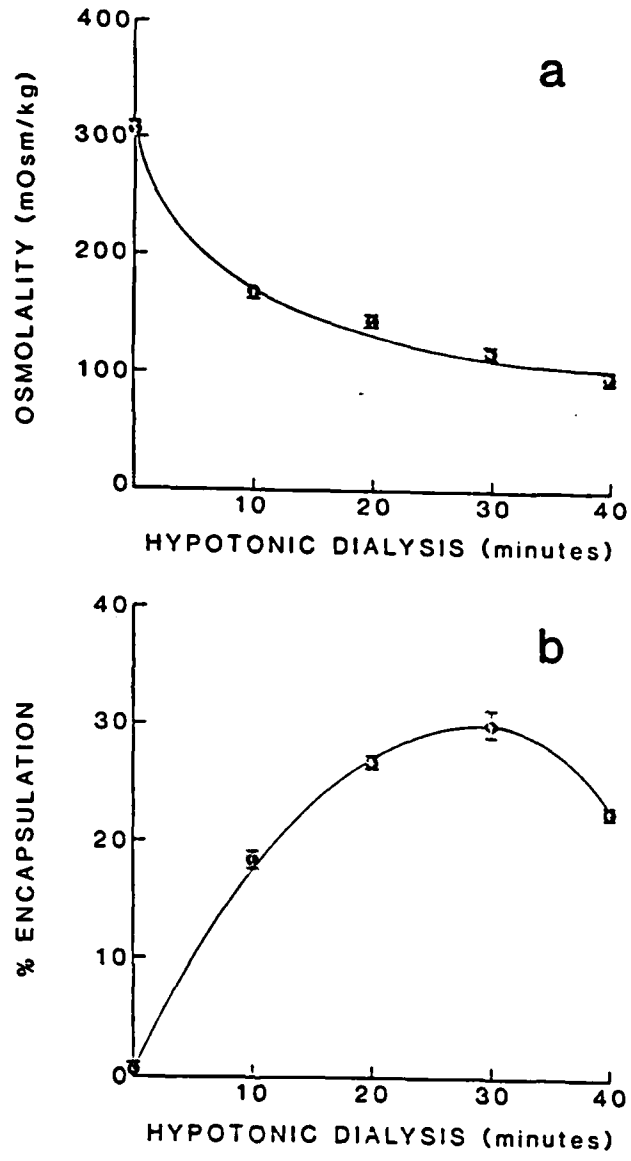
a
OSMOLALITY (mOsm/kg)
400
300
200
100
0
10
20
30
40
HYPOTONIC DIALYSIS (minutes)
b
% ENCAPSULATION
40
30
20
10
0
10
20
30
40
HYPOTONIC DIALYSIS (minutes)

FIGURE 2

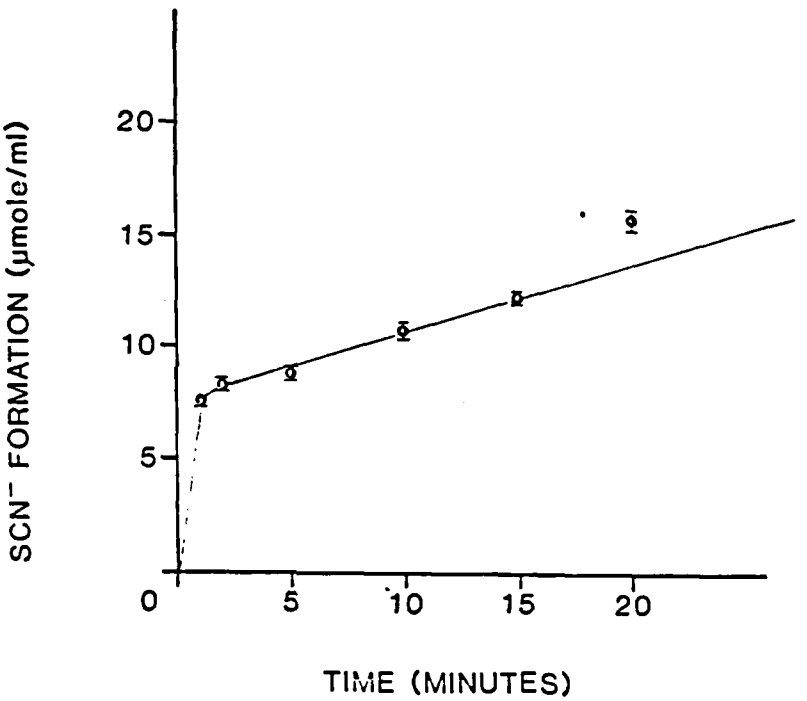

FIGURE 3

TABLE 1

RBC ENCAPSULATION

	Percent Encapsulation
^{3}H-Inulin	31.1 ± 4.8
^{14}C-Sucrose	30.1 ± 1.8
Rhodanese	30.1 ± 2.4

TABLE 2

ENCAPSULATION OF ^{14}C-SUCROSE IN MOUSE ERYTHROCYTES

	Erythrocytes	
	Dialyzed	Control
Encapsulation (%)	28.25 ± 3.88	0.69 ± 0.06
Equilibration (%)	86.69 ± 5.76	4.93 ± 0.38
Cell Recovery (%)	89.00 ± 2.08	96.03 ± 0.79
MCV (fl)	34.33 ± 0.08	40.13 ± 0.33
MCHb (pg)	13.99 ± 1.20	25.50 ± 0.24

TABLE 3

ENCAPSULATION OF THIOSULFATE : CYANIDE SULFURTRANSFERASE IN MOUSE ERYTHROCYTES

	Erythrocytes	
	Dialyzed	Control
Encapsulation (%)	29.63 ± 0.72	4.65 ± 0.26
Equilibrium (%)	84.30 ± 3.95	4.71 ± 0.05
Cell Recovery (%)	94.47 ± 2.30	99.03 ± 1.61
MCV (fl)	35.52 ± 0.06	40.13 ± 0.33
MCHb (pg)	11.96 ± 0.15	18.85 ± 0.13

END

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